RGT 7033

DNA Composition and Uses Thereof

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Related Application

This application is a continuation of USSN 60/440,245 filed January 15, 2003. A corresponding PCT Application, Atty Docket No. RGT 7033, is filed concurrently herewith. Both applications are incorporated herein by reference as if set forth in full.

Field of the Invention

The present invention relates to improved DNA sequences that can be used to induce immune responses, and methods for the treatment and prevention of infectious and neoplastic diseases. A plasmid DNA that encodes one or more antigenic genes operably linked to a promoter and a truncated 3' LTR derived from human immunodeficiency virus exhibits both enhanced safety and acceptable efficiency of expression of antigenic proteins.

Background of the Invention

Every form of classical vaccine, namely the use of killed virus, live attenuated virus, and various combinations of subunits of virus, has been tried for the prevention of HIV infection, to no good effect. It had been found that the classical vaccines worked as intended, but that the resulting immune response, an antibody response, was fundamentally unable to inhibit infection in animal models.

The present inventors have shown that, if an antigen is taken up by immune system cells and expressed in the lymphoid organs, different immune responses can be raised, and might have the potential to control the virus. See USSN 08/803,484 "Methods and Compositions for Therapeutic and Genetic Immunization, filed February 20, 1997 by J. Lisziewicz and F. Lori, Atty docket No. RGT-100A. The text of that application is incorporated by reference herein as if set forth in full. That application further disclosed that, while any number of antigens, including viral particles, might be used to raise the immune responses, it would be preferable to use a replication-defective virus due to safety considerations. The application explains that replication-defective viral particles themselves have been shown to be ineffective for raising immune responses. Rather than use viral particles, the inventors preferred to use a different material, a plasmid DNA encoding a replication-defective virus. The application discloses various types of replication-defective viruses to select for encoding, and suggests various ways to obtain replication-defective retroviruses, in particular by alteration of the integrase and gag genes. The inventors have demonstrated that a plasmid DNA encoding a replication-defective virus can be made used to induce immune responses, and that furthermore, such materials can be administered topically without the use of needles See USPN 6,420,176, Method of Delivering Genes into Antigen Presenting Cells, issued July 16, 2002 to Lisziewicz and Lori. The text of this patent is incorporated herein as if set forth in full.

The inventors have also demonstrated that plasmid DNA encoding a replication-defective virus having an altered integrase gene can be used in conjunction with an effective antiviral drug regimen to treat an existing infection. In a situation where antiviral drugs are able to control an infection but not eradicate it, the drugs can be

used until the virus replication is controlled, and then the patient can be treated with the plasmid DNA composition. After this therapeutic vaccine treatment, the patient may exhibit enhanced immune system function, and decreased need for drug treatment. (See USSN 09/863,606 "Therapeutic DNA vaccination," filed May 23, 2001 by Lisziewicz and Lori, Atty docket No. 7028). The examples of that application use an experimental plasmid DNA that was not intended for use in humans.

The inventors now describe a new plasmid DNA for the induction of T cell-mediated immune responses, which can be used to make improved DNA formulations. The plasmid DNA comprises one or more DNA sequences encoding antigenic materials operably linked to a promoter and also having a truncated 3' LTR. The promoter is preferably a 5' LTR. The truncated 3' LTR is important because this feature alone renders any retrovirus encoded by the DNA replication-defective. These DNA can be administered to mammals by any of the previously described methods. However, the inventors prefer topical administration, as this method is both efficient and comfortable for the patient.

Plasmid DNA has been used by both the present inventors and others for induction of immune responses against different pathogens, including HIV. For example, US 6,214,804 describes the use of a different DNA composition, specifically a DNA encoding the protein gp120 operably linked to a CMV promoter. As discussed herein, a CMV promoter is a constitutive promoter, which is less efficient than an inducible promoter. Further, there is no disclosure or discussion of the use of a truncated 3' LTR, or the advantages that may be obtained by its use.

In another example, WO 99/43350 (PCT/US99/04128) describes the use of ADP-ribosylating exotoxins as vaccine adjuvants. An advantage of the present invention is that the formulation can be fully comprised of materials having very low toxicity. In yet another example, US 6,348,450 B1 describes the use of adenovirus vectors as adjuvants in combination with DNA vaccines. The reference discloses that such vectors are immunogenic, and are of limited utility where the target individual has already been exposed to such vectors. An advantage of the present invention is that

it need not rely on the use of any immunogenic adjuvants. Therefore, unwanted immunogenic responses are minimized.

Other references disclose the use of DNA vaccines in animals. In particular, primate animal models are widely used for studies involving new preventive and therapeutic approaches for HIV infection (Robinson, H. L. (2002). "New hope for an AIDS vaccine." Nature Rev Immunol **2**(4): 239-50.). In this reference some of the DNA vaccines administered in combination with other vaccines prior to infection have demonstrated inhibition of viral replication after challenge (Robinson 2002). All of the previously described DNA vaccine constructs had a sequence composition of expressing one or more HIV genes operably linked to CMV promoter. None of these constructs were operably linked to an HIV 5' LTR promoter and none of these constructs were operably linked to a truncated 3' LTR. In our previous applications (PCT US97/02933) we have described a novel composition of DNA vaccine that expresses of replication defective viruses that expresses viral genes operably linked to 5' LTR promoter. In this application we further specify the composition of the DNA by operably linking one or more genes to the truncated 3' LTR and a promoter.

Brief Description of Drawings

Fig. 1 Composition of the nucleotide sequence of LW (Sequence Id. No.1). Location of 5' and 3' LTR, genes encoding all HIV proteins and regulatory sequences (TAR, RRE) are indicated.

Fig. 2 Composition of the nucleotide sequence of pLWXu1 (Sequence Id. No. 2). This DNA contains sequences required for propagation in E. coli as well as the kanamycin resistance gene originating from pNGVL3. Location of 5'LTR and the truncated 3' LTR, genes encoding the wild type regulatory proteins (tat, rev, vpr, vpu, vif) and structural proteins (env, reverse transcriptase, gag, protease), mutant integrase and nef and regulatory sequences (TAR, RRE) are indicated.

Fig. 3 Detail of pLWXu1 from Fig. 2, showing truncation of the 3' LTR.

Fig. 4 Diagram of complete inactivation of reverse transcription and integration by the truncated 3' LTR. Example is based on the plasmid pLWXu1. Thick line: DNA; thin line: RNA (Fields, B. N. and D. M. Knipe, Eds. (1990). Virology. Retroviridae and their replication. New York, Raven Press, LTD).

Fig. 5 Composition of the nucleotide sequence of pLWXu2 (Sequence Id. No. 3). The difference between pLWXu1 and pLWXu2 is that pLWXu1 contains a wild-type gag gene and pLWXu 2 contains a mutant gag gene. The mutant gag gene is dominant negative mutant able to inhibit HIV replication.

Fig. 6. Compares of gene expression of a prior art integrase mutant DNA (pLWint4K) and new pLWXu1. Both plasmid DNA express HIV proteins efficiently as measured here by quantitative p24 ELISA. The columns represent the median of 3 transfections of T293 cells using the formulated product. Two columns represent 2 independent experiments performed at different time points. Plasmid encoding the green fluorescent protein (GFP, no expression) was used as the negative control.

Fig. 7 Compares the way different methods for DNA immunization penetrate the skin to various degrees. Langerhans cells can be found very close to the surface, just under the stratum corneum. Topical administration most closely targets the position of the Langerhans cells.

Fig. 8 Compares the composition of replication- and integration-defective human immunodeficiency virus (HIV)(See LW, Sequence Id. No. 1) and a similarly-modified simian-human immunodeficiency virus (SHIV)(Sequence Id. No. 4).

SHIV plasmid DNA was constructed to demonstrate antiviral efficacy using a DNA encoding a replication- and integration-defective virus in SIV-infected macaque model. The HIV construct contains similar mutations in the integrase gene.

Fig. 9 Describes the mechanism of induction of immune responses after topical immunization using DNA/PEIm/glucose formulation.

Summary of the invention

The present invention relates to improved DNA sequences that can be used to induce immune responses, and methods for the treatment and prevention of infectious and neoplastic diseases. A plasmid DNA that encodes one or more antigenic genes operably linked to a promoter and a truncated 3' LTR derived from human immunodeficiency virus exhibits both enhanced safety and acceptable efficiency of expression of antigenic proteins. The compositions of Sequence Id. Nos. 2 and 3 are particularly preferred for raising an immune response against HIV, as exhibiting enhanced safety due to multiple mutations that do not substantially interfere with efficiency of expression of antigenic proteins. An advantage of the most preferred embodiments is that the constructs mimic the expression and antigen presentation of the wild-type HIV by using an inducible promoter compatible with the target cells and preserving most regulatory genes. An exception is the *nef* gene, which is substantially preserved for the purpose of producing an immune response, but which is also mutated in a form taken from pediatric long-term survivors. Another advantage is that the preferred embodiments completely inactivate the integrase gene in a manner designed to avoid replication and integration of any potential new viral particles and also avoids the risk that a replication competent virus might emerge from mutation of the constructs or interaction with the patient's own HIV. Yet another advantage is that the most preferred forms of the genes that are used do not exhibit drug resistance. Other benefits and advantages of the present invention will be apparent from the text and examples contained herein.

Detailed description of the invention

Description of the DNA construct

In one embodiment, this invention is a DNA construct designed to induce immune responses. The DNA contains one or more genes operably linked with a promoter and has a truncated 3'LTR.

The promoter can be any promoter capable of promoting gene expression in mammals, including for example, constitutive promoters such as CMV and SV-40. Generally constitutive promoters, if they function, will always promote the same level of expression of the affected genes. Inducible promoters are another class, where gene expression is subject to more detailed control. Inducible promoters require the present of some stimulus in order to act. That is, the promoter will not induce gene expression (or will induce very little expression) unless it is activated by the inducer. Further, inducible promoters may be sensitive to the concentration of the inducer. That is, if more of the inducer is present, more gene expression will result. In the preferred embodiment, a promoter induced by the Tat protein, or Tat-inducible promoter, is used. Most preferred is an LTR promoter derived from HIV (HIV-LTR). The LTR does not have to be full length. That is, the 5'end of the LTR can be deleted because it contains only the NRE (negative regulatory elements). The 5' LTR should express gene products efficiently after Tat activation. The advantage of the LTR promoter is that it has a nice balance of efficiency and safety. Tat-induced gene expression is generally more efficient than expression induced by constitutive promoters. However, in the absence of Tat protein there is no (or very low levels of) expression of the genes operably linked to LTR. This mechanism of regulated gene expression mitigates the potential side effects that might originate from a constitutive expression of genes. That is, both the timing and amount of gene expression is more favorably controlled. Another advantage of using Tat-inducible LTR in the present invention is that Tat-inducible LTR is known to be compatible with dendritic cells, and so it a likely candidate to function in a predictable fashion. Further, Tat is also immunogenic, and may contribute to the vaccine's effectiveness.

The preferred DNA construct also contains a truncated 3' LTR. Normally, a retrovirus has two promoters designated by their locations, 5' and 3'. Normally, 3' LTR functions as a promoter of DNA synthesis in retroviruses. In the present invention, 3'LTR is truncated at least enough to disable it from serving as a promoter. The mutation in the 3'LTR region is important for the safety of the DNA constructs for human use. The most important deletion in the 3' LTR is the deletion of the R region because in the absence of this region reverse transcription and integration of the viral DNA cannot take place (Fig. 3). In the preferred embodiment the deletion involves the R and U5 regions, TATA signal, and also the SP1 and NF-kappaB enhancers (Fig. 3).

The preferred DNA construct encodes genes for immunogenic proteins. Immunogenic proteins originate from viruses, such as HIV, HTLV, Herpes Viruses, influenza viruses, Hepatitis B virus, Hepatitis C virus, Human Papilloma Virus, and from tumors, including oncogens such as MAGE. In one embodiment these genes originate from HIV, and are selected from wild type and mutant versions of the tat, rev, nef, vif, vpr, vpu, env, gag, int, protease, and reverse transcriptase genes. Those of ordinary skill in the art are aware that, in the case of HIV, the genes are highly variable, and that different forms of HIV, called clades, are prevalent in different parts of the world, and may be present, or change from time to time, in a single individual. Therefore, a wide variety of mutations may occur in these genes. The most desired characteristic of these genes is that they contain one or more epitopes of the encoded proteins. An epitope is the minimal portion of an immunogenic protein that is capable of inducing an immune response.

As described above, the genes in the DNA construct can encode mutant proteins. Mutant proteins can be selected to decrease the toxicity or side effects from the wild type protein; improve the efficacy of the DNA construct, or further improve other safety features of the DNA construct.

For example, the wild-type nef protein is known to down-regulate CD4 expression, and may impair immune responses. That is, the wild-type nef protein exhibits toxic effects. Mutations in the nef gene can be selected to decrease the toxicity of the

DNA construct. If useful epitopes of mutant nef gene can still be expressed, immune responses against the nef protein can be at least partially preserved.

In another example, the introduction of a dominant negative mutant in the DNA can improve its efficacy, because the expression of such mutant proteins not only induces immune responses but also inhibits virus replication. This class of mutations was originally described for gene therapy purposes and not for the induction of immune responses. However, dominant negative mutant proteins, by definition, inhibit the function of the wild-type protein. Therefore, introduction of dominant negative mutations in one or more genes of the present DNA construct could not only preserve the immune responses against the encoded proteins but also interfere with the replication of the target virus. This new use of dominant negative mutants could be particularly important when the objective is being used to treat an existing infection rather than merely prevent infection. In DNA constructs whose purpose is to induce immune responses against HIV, dominant negative mutants are known, and can be selected at least from the following genes:

i. Envelop (Chen, S. S., A. A. Ferrante, et al. (1996). "Characterization of an envelope mutant of HIV-1 that interferes with viral infectivity." Virology 226(2): 260-8)
ii. Gag (Smythe, J. A., D. Sun, et al. (1994). "A Rev-inducible mutant gag gene stably transferred into T lymphocytes: an approach to gene therapy against human immunodeficiency virus type 1 infection." Proc Natl Acad Sci U S A **91**(9): 3657-61)
iii. Integrase (Yung, E., M. Sorin, et al. (2001). "Inhibition of HIV-1 virion production by a transdominant mutant of integrase interactor 1." Nat Med **7**(8): 920-6)
iv. Rev (Plavec, I., M. Agarwal, et al. (1997). "High transdominant RevM10 protein levels are required to inhibit HIV-1 replication in cell lines and primary T cells: implication for gene therapy of AIDS." Gene Ther **4**(2): 128-39)
v. Tat (Fraisier, C., D. A. Abraham, et al. (1998). "Inhibition of Tat-mediated transactivation and HIV replication with Tat mutant and repressor domain fusion proteins." Gene Ther **5**(7): 946-54)

vi. Vpr (Sawaya, B. E., K. Khalili, et al. (2000). "Transdominant activity of human immunodeficiency virus type 1 Vpr with a mutation at residue R73." J Virol **74**(10): 4877-81)

vii. Tax (Gitlin, S. D., P. F. Lindholm, et al. (1991). "Transdominant human T-cell lymphotropic virus type I TAX1 mutant that fails to localize to the nucleus." J Virol **65**(5): 2612-21)

viii. Rex (Bohnlein, S., F. P. Pirker, et al. (1991). "Transdominant repressors for human T-cell leukemia virus type I rex and human immunodeficiency virus type 1 rev function." J Virol **65**(1): 81-8)

ix. X25 (Smith, C. A. and N. A. DeLuca (1992). "Transdominant inhibition of herpes simplex virus growth in transgenic mice." Virology **191**(2): 581-8)

x. Mutant ICPO (Chen, J., C. Panagiotidis, et al. (1992). "Multimerization of ICP0, a herpes simplex virus immediate-early protein." J Virol **66**(9): 5598-602)

Mutant proteins in the DNA construct can also significantly improve the safety of the use of this DNA in human subjects. One safety concern when introducing DNA in virus-infected subjects is that recombination that can theoretically occur between the DNA containing viral genes and the virus. This recombination might generate recombinant replication competent viruses. Using DNA constructs with mutant viral genes can eliminate this risk, because recombinant viruses containing a dominant negative mutation are not only replication-defective but also can inhibit the replication of wild-type viruses.

Description of the preferred embodiment of the DNA construct

In the preferred embodiment the DNA construct is comprised of nucleotide sequences selected from LW (Fig. 1 and Sequence Id. No.1). This construct is preferred for a number of reasons. It contains all of the HIV genes, arranged in the manner of the wild-type virus, so that a broad immune response can be raised, thereby limiting the possibility that the virus might avoid immune system recognition by developing escape mutations. These genes do not contain mutations that confer drug resistance. This is important, as a factor that might preserve the utility of drug treatments. Evidence that this is so can be found in referenes that show that the

replication of LW virus can be effectively blocked by conventional antiretroviral therapy (Piccinini, G., A. Foli, et al. (2002). "Complementary antiviral efficacy of hydroxyurea and protease inhibitors in human immunodeficiency virus-infected dendritic cells and lymphocytes." J Virol **76**(5): 2274-8). This DNA does not contain artificial sequences created in the laboratory, such as codon optimized sequences, so that authentic function has been preserved. Finally, all of the immunogenic proteins expressed by pLW are abundantly expressed in HIV-infected subjects. As a result, the amount of expression of these proteins by the construct is expected to be trivial compared to that of the wild-type virus, and so no new, harmful materials are introduced into the patient by the vaccine.

The promoter

The promoter in the preferred embodiment is the 5'LTR selected from the sequence of LW (Sequence Id. No.1). The 5'LTR is located between nucleotides 1 and 630.

Truncated 3' LTR

The truncated 3' LTR in the preferred embodiment is selected from the sequence of LW (Sequence Id. No.1). The truncation is depicted at the Fig. 3. The truncation of the 3' LTR starts at nt. 9244 in the LW sequence.

Mutant nef gene

The preferred truncation of the 3' LTR also results in truncation of the nef gene (Fig. 3), which results in expression of a mutant Nef protein. However, this mutant nef gene is still capable of expressing immunogenic epitopes of Nef, so the DNA constructs containing this mutant are suitable for the induction of Nef-specific immune responses.

Mutant integrase gene

The mutant integrase gene in the preferred embodiment is selected from the sequence of LW (Sequence Id. No.1). The mutation is depicted at Fig. 2. The specific mutations include STOP codons in the open reading frames and a seven base pair deletion. These genetic modifications were carefully designed to avoid potential reversion of the plasmid DNA to the wild type virus. A total of six stop codons were created in the open reading frame of the integrase gene; two by base pair mutations and four by the seven base pair deletion. In addition, the seven base

pair deletion created a potential stop codon in the shifted open reading frame and a non-integrase protein product in the other shifted open reading frame. It is highly unlikely that all the six stop codons could simultaneously mutate *in vivo* and revert to wild type sequences. Even if these six simultaneous mutations were to happen, functional integrase protein would not be reconstituted due to the shifted open reading frame resulting from the seven base pair deletion. This mutant integrase gene is still capable of expressing immunogenic epitopes of integrase, therefore the DNA constructs containing this mutant are suitable for the induction of integrase-specific immune responses.

Mutant gag gene

The mutant gag gene in the preferred embodiment is selected from the sequence of LW (Sequence Id. No.1). The mutation is depicted at Fig. 5. The specific mutation includes a deletion in the protease cleavage site between p17 and p24. This deletion results in a mutant gag protein that will be incorporated into the viral particle. However, in the absence of protease cleavage virus core and envelope are not separated and the mature viral particle cannot be released. Regarding immunogenicity, the mutant gag still expresses most of the immunogenic epitopes of the gag protein, therefore DNA constructs containing this mutant are suitable for the induction of gag-specific immune responses.

Expression of structural and regulatory HIV proteins

The DNA construct in the preferred embodiment comprises of wild-type structural genes (envelop, reverse transcriptase, protease) and wild-type regulatory genes (tat, rev, vpr, vpu, vif). Expression of these genes can induce immune responses directed against all the epitopes of these genes, therefore the DNA construct is suitable for the induction HIV-specific immune responses with broad specificity.

Detailed description of the plasmid pLWXu1

The parental plasmid pLWint4k has already been shown to be both replication-defective and also capable of inducing therapeutic virus-specific immune responses. Because this vaccine is intended for use in humans, further modifications to enhance safety are desired, particularly if these can be achieved without significant loss of efficacy. New plasmid pLWXu1 is pLWint4k with a number of modifications that

interrupt the viral life cycle at several points and minimize the probability of reverse transcription, integration, reversion or recombination with the host's HIV-1.

As is the case with pLWint4k, in pLWXu1 the viral gene expression is preferably driven by the 5' HIV-1 LTR, a *tat*-inducible promoter. A promoter is essential for the efficacy of the product, that is, for efficient expression in dendritic cells and authentic expression and processing of viral proteins, and therefore if no promoter were present the efficacy and the benefit of this therapeutic vaccine will be lost. However, other promoters might be used to replace 5' LTR with similar efficacy.

In human cells, initiation of transcription of the viral RNA is induced by cellular transcription factors (e.g. NFkB). This RNA is transported from the nucleus to the cytoplasm through splicing and these multiple spliced mRNAs are the templates for translation of small viral regulatory proteins (e.g. Tat, Rev, Nef). When the Tat protein translocates back to the nucleus it further activates the transcription of viral RNA.

After efficient transcription in the nucleus, RNA transport to the cytoplasm occurs authentically. First, multiple splicing takes place to produce the mRNA for the regulatory genes. Early expression of these regulatory proteins ensures rapid antigen presentation and initiation of potent immune responses against these less abundant HIV proteins.

After expression of early regulatory proteins Rev transports singly spliced or unspliced RNA to the cytoplasm. These mRNAs encode the structural proteins, Gag, Pol and Env. Antigen presentation of epitopes derived from the most abundant structural proteins therefore occurs later.

CMV or other constitutive promoters cannot achieve in a qualitative and quantitative sense in the following aspects of transcriptional and translational regulation, which are essential for the efficacy of this therapeutic vaccine product:

- High level of gene expression. It is known that the HIV-LTR is a stronger promoter in the presence of Tat than CMV (Jayan, G. C., P. Cordelier, et al. (2001). "SV40-derived vectors provide effective transgene expression and

inhibition of HIV-1 using constitutive, conditional,and pol III promoters." Gene Ther **8**(13): 1033-42).

- Gene expression in dendritic cells. It has been demonstrated *in vivo,* in HIV-1 infected individuals, that HIV-LTR can express genes in dendritic cells (Frankel, S. S., K. Tenner-Racz, et al. (1997). "Active replication of HIV-1 at the lymphoepithelial surface of the tonsil." Am J Pathol **151**(1): 89-96).
- We believe that the timing of viral antigen presentation (e.g. early expression of regulatory proteins) is very important for the efficacy of the product.

All the regulatory proteins are intact, except the *nef* gene, which is truncated at the 3' end (Fig. 3). This deletion does not eliminate the major immunogenic epitopes but improves the safety features of the vaccine product. It has been shown that Nef is required for the pathogenicity of HIV-1 (Jamieson, B. D., G. M. Aldrovandi, et al. (1994). "Requirement of human immunodeficiency virus type 1 nef for in vivo replication and pathogenicity." J Virol 68(6): 3478-85; Aldrovandi, G. M., L. Gao, et al. (1998). "Regions of human immunodeficiency virus type 1 nef required for function in vivo." J Virol 72(9): 7032-9). Defective *nef* genes have been found in pediatric long-term survivors (Geffin, R., D. Wolf, et al. (2000). "Functional and structural defects in HIV type 1 nef genes derived from pediatric long-term survivors." AIDS Res Hum Retroviruses 16(17): 1855-68).

The pLWXu1 plasmid expresses a completely inactivated integrase gene. Integrase is an essential gene for virus replication, We have shown that mutations introduced into integrase gene block viral integration and produces a virus that is not capable of replication and integration (Lisziewicz, J., D. I. Gabrilovich, et al. (2001). "Induction of potent human immunodeficiency virus type 1-specific T-cell-restricted immunity by genetically modified dendritic cells." J Virol **75**(16): 7621-8).

We have introduced an extensive 3' U3 deletion (Fig. 2 and 3) that abolishes viral promoter activity in 3' LTR, thereby preventing the synthesis of a cellular gene located downstream from the 3' LTR (Zufferey, R., T. Dull, et al. (1998). "Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery." J Virol 72(12): 9873-80).

We have deleted the 3' R region and consequently completely impaired reverse transcription (Fig. 2 and Fig. 3), thereby preventing viral DNA synthesis in infected cells. This deletion eliminates the risk that a replication competent virus will emerge from the pLWXu1 plasmid. Importantly, this mutation ensures a higher level of safety than mutating the gene encoding the viral reverse transcriptase (RT) because this defect cannot be rescued *in trans* by the patient's own HIV.

We have deleted the U5 region, which contains one of the att sites necessary for viral integration (Vicenzi, E., D. S. Dimitrov, et al. (1994). "An integration-defective U5 deletion mutant of human immunodeficiency virus type 1 reverts by eliminating additional long terminal repeat sequences." J Virol 68(12): 7879-90; Zhou, H., G. J. Rainey, et al. (2001). "Substrate sequence selection by retroviral integrase." J Virol 75(3): 1359-70). Besides further decreasing the risk of integration by viral integrase *in trans*. That is the risk that another virus in the host cell might combine with the vaccine's plasmid or resulting viral particle and supply a functional integrase gene, thereby resulting in the unwanted reacquisition of the ability to integrate. Further, the theoretical possibility of integration by alternative pathways mediated by "integrase-like" enzymes of the host has also been excluded. There is evidence that this region contains essential elements for packaging of viral RNA (Murphy, J. E. and S. P. Goff (1989) "Construction and analysis of deletion mutations in the U5 region of Moloney murine leukemia virus: effects on RNA packaging and reverse transcription." J Virol 63(1): 319-27), therefore the risk of packaging of RNA derived from pLWXu1 is also decreased.

Due to the various mutations described above, pLWXu1 has improved safety compared to parental plasmid pLWint4k. The improvement of safety features in pLWXu1 does not sacrifice the efficacy of gene expression, because the production of antigenic proteins is very similar, as shown in Fig. 6, which compares p24 expression after transfection of the two plasmids to T293 cells.

Vector containing the bacterial genes

In order to manufacture the DNA in bacterium, the DNA must comprise bacterial sequences required for propagation of the DNA. Since the DNA construct is designed for human use, the Kanamycin gene is inserted for use as a selection marker. In the preferred embodiment the Kanamycin gene and the other bacterial

genes were derived from a known vector (pNGVL3) and the CMV promoter sequence present in the original pNGVL3 vector was deleted during the subcloning process (see pLWXu1 at Fig. 2 and Sequence Id. No. 2).

Advantages of the pLWXu1 DNA construct

This plasmid DNA shows antiretroviral activity, as a result of its features specifically designed for the induction of HIV-specific immune responses:

1. Authentic expression of HIV genes and authentic antigen presentation by dendritic cells to naïve T cells mimics 'autovaccination' (Lisziewicz, J., E. Rosenberg, et al. (1999). "Control of HIV despite the discontinuation of antiretroviral therapy." N Engl J Med 340(21): 1683-4; Lori, F., M. G. Lewis, et al. (2000). "Control of SIV rebound through structured treatment interruptions during early infection." Science 290(5496): 1591-3; Rosenberg, E. S., M. Altfeld, et al. (2000). "Immune control of HIV-1 after early treatment of acute infection." Nature 407(6803). The expression of the DNA construct mimics the expression and antigen presentation of wild type HIV, which we believe is essential to provide maximum therapeutic effectiveness.
2. 5' HIV-LTR as the promoter: LTR-driven gene expression is an important feature of the construct to mimic the effect of "autovaccination". This promoter is utilized for viral gene expression and antigen presentation by dendritic cells during primary HIV infection. Moreover, exchanging the LTR to a heterologous promoter (e.g. CMV) would significantly decrease the effectiveness of gene expression.
3. 3' HIV-LTR: This contains an immunogenic part of the nef gene and the transcription stop sequence required for gene expression.
4. Expression of regulatory genes (tat, rev, nef, vpr, vpu, vif): These genes are expressed early, about 24 hours prior to virion production, in infected cells. Cytotoxic T cells that can eliminate infected cells prior to virion production are essential ingredients of a therapy, which is based on inducing HIV-specific T cell immunity.
5. Expression of gag and pol genes: Most of the immunodominant epitopes are located in gag and pol.
6. Expression of tat, rev, vpr, vpu, vif, gag, pol: These genes are the most common between different clades of HIV-1. Immunity against these proteins is

not only important to provide antiretroviral activity against different clades of the virus, but also to minimize the chances of immune escape (Barouch, D. H., J. Kunstman, et al. (2002). "Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes." Nature 415(6869): 335-9).

7. Expression of env: The env gene is an important part of the construct because it contains a portion of the open reading frames of the regulatory genes. It also contains the RRE, which is essential for regulation of HIV gene expression in the construct. In addition, expression of env has been shown to improve the effectiveness of vaccines that inhibit virus replication after infection by priming env-specific T helper cells (Robinson, H. L. (2002) "New hope for an AIDS vaccine." Nature Rev Immunol 2(4): 239-50).

Other DNA vaccines developed for the prevention of HIV infection use humanized DNA constructs to encode one or more HIV genes expressed by a heterologous promoter (e.g. CMV). These constructs could introduce new DNA sequences into humans with unknown pathogenic consequences. In contrast, the DNA construct described here allows expression of most HIV antigens in dendritic cells without the need for codon optimization (humanization).

Detailed description of the plasmid pLWXu2

Plasmid pLWXu2 is derived from pLWXu1. pLWXu2 contains an additional deletion mutation in the gag gene (Fig. 5). Consequently, pLWXu2 contains all the safety features of pLWXu1 as described above. The specific mutation in gag gene of pLWXu2 includes a deletion (from nucleotides 1097 to 1267 in Sequence Id. No.1) at the protease cleavage site between p17 and p24. It has been demonstrated that the deletion alone could block the release of HIV virions from the infected cells, thereby interrupting the viral life cycle (Trono, D., M. B. Feinberg, et al. (1989). "HIV-1 gag mutants can dominantly interfere with the replication of the wild-type virus." Cell 59: 113-120.). Therefore, the introduction of the said deletion into gag gene further improves the safety features of pLWXu2.

We found that, the gag deletion resulted in the loss of at least one p24 epitope recognized by ELISA antibody (*via* Coulter HIV p24 antigen assay), which excludes the determination of gag expression with ELISA. Consequently, a flow cytometry

assay using another antibody (KC57, Coulter staining both p24 and its precursor p55 proteins) was employed to determine the expression of the gag gene in pLWXu2-transfected T293 cells. We found efficient HIV gag gene expression with both pLWXu1 and pLWXu2 by flow cytometric assay, suggesting that both of these plasmid DNAs can induce HIV-specific immune responses. However, as shown in Table 1 by the value of mean fluorescence intensity (MFI), pLWXu2 expresses lower amounts of p24 protein than pLWint4k and pLWXu1. It is unclear what is the mechanism that causes the lower amount of expression of the mutant gag in pLWXu2. One possibility is that the mutant gag is unstable and rapidly undergoes degradation, therefore less p24 can be detected by this assay. In this case, pLWXu2 might be more efficient to induce T cell immune responses than pLWXu1 because it has been shown that targeting a protein to a degradation pathway can improve the resulting antigen presentation and T cell activation (Grant, E. P., M. T. Michalek, et al. (1995). "Rate of antigen degradation by the ubiquitin-proteasome pathway influences MHC class I presentation." J Immunol 155(8): 3750-8).

Table 1: Mean fluorescence intensity of p24 staining in transfected T293 cells.

Plasmid used to transfect T293 cells	Mean Fluoresence Intensity in experiment 1	Mean Fluoresence Intensity in experiment 2
No plasmid	No positive cells	No positive cells
pLWint4k	142.5	86.4
pLWXu1	71.3	47.7
pLWXu2	19.7	8.9

Formulation of the DNA construct

The DNA construct invented here is designed to elicit immune responses. Therefore, this DNA is formulated in a pharmaceutically acceptable composition for the use of induction of immunity. The formulations of the disclosed nucleotide sequences include, but not limited to the following list:

1. Plasmid DNA in water solution
2. Plasmid DNA in physiological salt solution
3. Plasmid DNA in sugar (including glucose) solution
4. Plasmid DNA with transfection facilitating formulation
    a. In complex with viral vectors

b. Associated with liposomes
c. Associated with virosomes
d. In complex with PEI and derivatives (e.g. PEIm)
e. On gold particles
f. In cream
g. In suppository
h. In pills
i. Formulated to form particles
j. Formulated to form particles targeting specific cell types
    i. Formulated to target Langerhans cells
    ii. Formulated to target dendritic cells

5. The disclosed nucleotide sequences can be introduced to viral vectors
    a. Adenovirus vectors
    b. Herpes virus vectors
    c. Adeno-associated virus vectors
    d. Retrovirus vectors
    e. Lentivirus vectors
6. The disclosed nucleotide sequence and the DNA constructs can be introduced into bacteria (bacterial drug formulation)

Preferred formulation of the DNA construct is the DermaVir formulation

We have developed and tested a DNA formulation in primates, and demonstrated the feasibility of using the DNA in combination with presently approved antiretroviral drugs. The preferred formulation ("DermaVir") consists of a plasmid DNA construct, polyethylenimine-mannose (PEIm) and dextrose in a water solution.

PEIm, molecular weight = 25-28 kD, is an organic macromolecule with a high cationic-charge-density potential. PEIm complexes the plasmid DNA and forms a particle. This particle mimics a bacteria, because it has a mannosilated surface and can both target epidermal Langerhans cells and facilitate gene expression by those cells.

PEIm is manufactured in five distinct steps or stages:

Step 1. Polymerization of 2-ethyl-2-oxazoline into Poly(2-ethyl-2-oxazoline) (PEOX)

Step 2. Purification of Poly(2-ethyl-2-oxazoline)

Step 3. Conversion of PEO to PEI and Purification of the PEI

Step 4. PEI derivatization to PEI-mannose

Step 5. Purification of PEIm

Analytical tests have been developed to characterize PEIm intermediate and end product, demonstrate PEI derivations and potency, and determine purity and relative amine concentration. Each batch of PEIm must pass eleven in-process analytical methods including NMR, Resorcinol-Sulfuric Acid, Gel permeation, endotoxin testing, and transfection of cells prior to release. Each PEIm bulk product lot is assigned a lot number, product number and a Certificate of Analysis.

10% Dextrose, USP

The formulation of DermaVir uses aqueous dextrose. 10% aqueous in 5 ml ampules (Abbott Laboratories, North Chicago, Illinois). The specification is 10% dextrose for injection, USP, a sterile, nonpyrogenic solution (NDC 0074-4089-02), containing no antimicrobial agent or added buffer.

DermaVir formulation

Three components of DermaVir:
Plasmid DNA in water solution (1 mg/ml),
PEIm (13.6mM),
Dextrose (10%).

Step 1. Prepare Solution A: Combine 0.2 ml PEIm with 0.6 ml dextrose, cap and invert 5 times

Step 2. Prepare Unit B: Combine 0.2 ml plasmid DNA with 0.6 ml dextrose, cap and invert 5 times

Step 3. Prepare DERMAVIR: Combine Unit A and Unit B, cap and invert 5 times.

Tests and Specifications for Quality Control of DermaVir formulation with pLWXu1

Procedure		Purpose	Method	Specification to Pass
Number	Title			
OP-2005	Transfection assay	Determination of the potency of DERMAVIR	Transfection of T293 cells with DERMAVIR	> 30 ng/mL of p24 antigen
OP-2004	Stability assay	Determination of the stability of DERMAVIR at room temperature	Transfection assay 24 hours after formulation of DERMAVIR	> 30 ng/mL of p24 antigen
OP-2007	Residual charge assay	Determination of the charge of DERMAVIR	Agarose gel electrophoresis	Complete retardation

The transfection assay is a suitable marker for the potency of the DermaVir formulation, because potency is directly dependent upon expression of the DNA by the transfected cells. The assay measures the potency of a formulation by quantifying a protein, HIV p24, found in the supernatant of a sample after transfection.

We used the pLWXu1 plasmid DNA in the DermaVir formulation, and tested it as follows. T293 cells are plated in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, l-glutamine, penicillin, and are removed with trypsin, washed, and plated in a 48-well polystyrene culture plate. Prior to transfection, the media is exchanged for DMEM containing 16 μl DermaVir. Two hours post-transfection, DMEM is refreshed and, after 48 hours culture, supernatants are collected and stored at -20°C. To determine the expression of the DNA, HIV-1 p24 antigen in the supernatants is measured by quantitative ELISA (Coulter HIV-1 p24 Antigen Assay, U.S. License No. 1185). The FDA has licensed this kit for use with tissue culture specimens. Results depicted in Fig. 6 show that pLWXu1 expresses HIV-1 p24 antigen in amounts similar to the control, an integrase mutant DNA, that has been previously shown to elicit immune responses. The pLWXu1, therefore, can be used for the prevention and treatment of HIV.

The Residual Charge Assay measures a feature of the DermaVir formulation that is critical to potency. To be effective, the DNA must form a neutrally charged particle in

PEIm/dextrose solution. The test is based upon the fact that neutralized DNA does not migrate from a well during agarose gel electrophoresis. Thus, if a DermaVir formulation does not migrate under a charge (total complexation), the test provides evidence that the DNA in that lot of product is neutral and the product properly formulated. The test involves electorphoresis of 0.01mL sample of DermaVir formulation in standard 0.8% agarose gel. To visualize the DNA the gels are stained with Ethidium Bromide and migration is measured against a standard.

To date, we have completed 24 hour stability testing of the DermaVir formulations. The DermaVir transfection assay was employed freshly after formulation (0 hours) and after incubation of DermaVir formulation for 24 and 48 hours at room temperature to determine the stability. We found that this formulation is stable at room temperature for 48 hours.

Methods of administration for the induction of immune responses

The DNA composition invented here is useful for the induction of immune responses in mammals, including human subjects. For this use the formulations of the DNA construct described above can be administered via all typical routes, including topical, intradermal, subcutaneous, intramuscular, oral, rectal, or vaginal. The topical route is most preferred. The vaccine may be administered by a variety of devices, including a single or bifurcated needle gene gun; particle bombardment device, or skinVac device (from Becton Dickinson Biosciences San Jose, CA 95131)

Preferred administration of the DNA construct is topical

It is well established that the injection of DNA capable of expressing immunogenic proteins, like HIV proteins, can induce immune responses. However, less data is available for topical administration. The preferred route of administration of the DNA+PEIm+glucose formulation is topical, because

1. The formulation targets Langerhans cells located under the stratum corneum, therefore needle injection would miss these cells (see Fig. 7).
2. Langerhans cells are the precursor of dendritic cells that migrate to the lymph nodes. Antigen presentation by dendritic cells is important for the induction of effective immune responses.

3. Topical administration of the formulation was not toxic in primates and swine, suggesting the absence of toxicities in human subjects.

After formulation the product should be applied on a prepared skin characterized by a perforated stratum corneum. The perforation allows the DermaVir particle better access the Langerhans cells, which are located just below. Perforation is mild removal of the outermost layer of skin cells, and can be achieved by shaving, clipping, stripping, exfoliating, rubbing, scarifying and scratching the skin. We have used the following method:

1. Identify the sites for vaccination. For example, the left and right upper back (trapezius/suprascapular region) and the ventral aspect of the proximal left and right thigh. Any other vaccination sites covered by skin or mucose can be also used.
2. Gently shave the vaccination sites using a disposable razor.
3. Disinfect the vaccination sites using 70% isopropyl alcohol swabs.
4. Gently abrade the skin surface at the vaccination sites. This could be done by rubbing a Buf-Puf™ exfoliating sponge repeatedly (50 times back and forth) over the area, applying light pressure but taking care not to break the skin. Use a new side of sponge for each site. Other exfoliation methods can be applied here as well, for example using a special device.
5. Apply Tegaderm™ adhesive to each vaccination site and immediately strip off in one quick movement to remove residual cell matter on the skin surface. Repeat if necessary (high dose vaccination) to cover whole area of vaccination site.
6. Repeat taping procedure at a 90° angle to the first taping with the same adhesive.
    a. Using the 1ml-syringe and needle draw the formulated DNA. Using the 1ml-syringe (without needle!), apply the vaccine to one site.
7. Distribute the liquid formulation evenly over the vaccination site using the tip of the syringe, taking care to avoid spillage beyond the vaccination site to ensure optimal dosing.
8. Cover the area with the nonabsorbent wound dressing.

Preferred dose of the DNA construct in adult and pediatric subjects

Quantitative composition of the dosages form is as follows:

Dosage	Comp siti n	Total Volume
L w d se (single application)	0.1 mg DNA + 0.1 ml 13.6 mM PEIm + 0.6 ml 10% dextrose in water	0.8 ml
Medium dose (single application)	0.2 mg DNA + 0.2 ml 13.6 mM PEIm + 1.2 ml 10% dextrose in water	1.6 ml
High dose (single application)	0.4 mg DNA + 0.4 ml 13.6 mM PEIm + 2.4 ml 10% dextrose in water	3.2 ml

For adult subjects a total of 0.8 ml issued per low dose DermaVir administration; i.e., 0.2 ml DermaVir per site (four sites per patient). For high dose administration 0.8 ml DermaVir is administered per site (four sites per patient) for a total of 3.2ml. For pediatric patients Low dose and medium dose is recommended.

The preferred size of the skin surface for vaccination is:

a. Low-dose vaccination: 40 square centimeters
b. Medium-dose vaccination: 80 square centimeters
c. High-dose vaccination: 160 square centimeters

Primate SIV infection and disease progression mimics HIV infection of human subjects

The progression of SIV_{251}-infection in rhesus macaques as been shown to be similar to, but faster than that of HIV infection in humans. Further, the response of SIV-infected macaques to variations in antiretroviral drug therapies closely follows that of humans. This animal model has been validated in previous studies (Lisziewicz, J., E. Rosenberg, et al. (1999). "Control of HIV despite the discontinuation of antiretroviral therapy." N Engl J Med **340**(21): 1683-4; Lori, F., M. G. Lewis, et al. (2000). "Control of SIV rebound through structured treatment interruptions during early infection." Science **290**(5496): 1591-3; Rosenberg, E. S., M. Altfeld, et al. (2000). "Immune control of HIV-1 after early treatment of acute infection." Nature **407**(6803): 523-6) and was therefore chosen as the best animal model to study the question of whether particular HIV/AIDS vaccines might have a therapeutic benefit for humans.

Derivation of a vaccine formulation

Various molecular clones of HIV and variations thereon are available for scientific study. One such clone, SHIV, was chosen for the primate studies described herein,

because it is the closest relative of HIV that causes pathogenic infection in macaques. In addition, SHIV contains an HIV envelope that is heterologous to the SIV_{251} challenge virus; consequently the data obtained using this model can presumably be translated to clinical situations with HIV in man.

A Simian/Human Immunodeficiency Virus (SHIV) construct, that is, a plasmid DNA encoding a Simian/Human Immunodeficiency Virus (SHIV) was made with the same molecular characteristics as the HIV-based plasmid DNA, and used to formulate a DermaVir vaccine (Fig. 8).

The SHIV molecular clone was developed by introducing a mutation in the SHIV integrase gene at the same position as the mutations described above for the HIV vaccine construct, pLWint4K(Sequence Id. No. 1). This yielded the plasmid DNA encoding a replication- and integration-defective SHIV construct pSHIV(int-1) (Sequence Id. No. 4) whose sequence had molecular characteristics very similar to the DNA constructs described in the preferred embodiment (pLWXu1)(Sequence Id. No. 2). Indeed, it contained a deletion, frame shift and three separated stop codons in the open reading frame of the integrase gene and also stop codons in the other reading frame of this region.

Studies in non-human primates with pSHIV(int-1), formulated with polyethylenimine-mannose (PEIm) and a glucose solution (as described above) demonstrated that this topical, therapeutic DNA immunization could induce SIV-specific T cell responses. The quantity of these T cells was associated with clinical, immunological and virological benefit during chronic infection and AIDS. We conducted two separate studies on 26 chronically-infected primates and an additional 10 macaques already showing signs of AIDS (USSN 09/863,606 and PCT/US02/16546) In one experiment, the monkeys were randomized to receive HAART, (3 weeks on HAART and 3 weeks off), HAART+vaccine and the vaccine alone. The best results were obtained by the HAART+vaccine group, which progressively controlled viral rebound during treatment interruptions from a median 33,860 copies/ml to <200 copies/ml. All treated cohorts survived significantly longer than the untreated controls. In another experiment, 10 macaques with AIDS started the HAART+vaccine treatment with a significantly higher viral load and suppressed viral rebound from a median of

4,292,260 to <200 copies/mL. Control of viral load in the absence of therapy was associated with augmented SIV-specific CD8 and CD4 T cells as measured by IFN-gamma intracellular cytokine assay. While these experiments do not constitute safety studies, topical DNA vaccination (more than 8 doses at the same skin site) did not show signs of toxic side effects.

These experiments have demonstrated that HIV-specific immune responses can be induced by topical DNA vaccination using the composition described here and that such therapy can result in an immunity that leads to suppression of virus replication. DermaVir, the proposed human therapeutic vaccine, should employ authentic expression of most viral genes and authentic presentation of most viral epitopes by dendritic cells. Consequently, DermaVir treatment by induction of T cells that can eliminate virus infected cells in reservoirs of HIV-infected patients and SIV-infected monkeys. Indeed, products using both pSHIV(int-1) and pLWXu1 meet this criteria. Since the SHIV construct demonstrated antiretroviral effectiveness in SIV-infected macaques, we intend to test the same approach using DermaVir in human subjects.

There will be various modifications, improvements and applications of the disclosed invention that will be apparent to those of skill in the art, and the present application is intended to cover such embodiments.